TECHNICAL MANUSCRIPT 4

APPARENT TOXICITY OF ASCORBIC ACID TO SERRATIA MARCESCENS

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ABSTRACT

Neutral solutions of ascorbic acid were found to be toxic to Serratia marcescens at low but not at high population densities. Investigations disclosed that the presence of trace amounts of copper was responsible for the apparent antibacterial effects of ascorbate. The copper entered the solutions from the distilled water and from the copper cans used as pipette containers.

Solutions of ascorbate plus copper were equally toxic under aerobic and anaerobic conditions. Their antibacterial effects were abolished by (a) metal-chelating agents or treatments; (b) anions or organic molecules, which formed relatively weak complexes with the metals of the first transition series; and (c) NH_4^+ or Fe^{+++} cations.

A hypothesis was developed that attributed the toxic effects of copper in ascorbate solutions to its oxidation-reduction reaction with postulated indispensable cellular Fe^{+++} atoms.

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I. INTRODUCTION

While testing the effects of various solutes upon the survival of bacteria during freeze drying we found that Serratia marcescens, in sparsely (but not in densely) populated cell suspensions, was killed by ascorbic acid. In these tests, plate count assays were made to determine the number of viable cells in suspensions containing 20×10^9 S. marcescens per milliliter and one per cent sodium ascorbate (one gram of ascorbic acid, adjusted to pH 7.0 with NaOH, per 100 ml). Two parallel assays were made upon each suspension, using water as a dilution fluid in one assay and using one per cent sodium ascorbate in the other. The dilution series made in water yielded anticipated levels of colony count upon the plates, but the dilutions made with ascorbate yielded no colonies. This unexpected antibacterial effect of ascorbate was not duplicated by the sodium salts of other hexuronic acids such as glucuronic and galacturonic.

Eddy and Ingram[1]* pointed out, in a review of the interactions between ascorbic acid and bacteria, that the findings of different workers in this field were often contradictory: ascorbic acid was sometimes bactericidal, sometimes bacteriostatic, and sometimes inert. The adverse effects of ascorbate upon bacteria have been ascribed to its reduction of the pH or oxidation-reduction potential of the growth medium; or to the presence of diketones or H_2O_2 produced by its auto-oxidation.[2-5] Ericsson and Lundbeck[6,7] observed a synergistic enhancement of toxicity in mixtures of ascorbate, peroxide, and copper salts. The bactericidal effects of such mixtures were considerably stronger in saline solutions than in body fluids such as serum, urine, ascitic or spinal fluid. In these studies, however, the antibacterial effects of ascorbate were not influenced by the viable cell population levels of the suspensions.

None of these observations afforded an explanation of the interaction between cell concentration and the toxicity of ascorbate we observed, so the phenomenon was investigated under more defined conditions.

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* See Literature Cited.

II. MATERIALS AND METHODS

A. MATERIALS

S. marcescens cells were obtained as pelletized frozen cell concentrates, prepared by techniques previously described[8] and stored at -78°C. The thawed pellets, containing about 100×10^{10} viable cells per milliliter, were diluted with distilled water to yield stock suspensions containing 40×10^{9} viable cells per milliliter. The stock suspension was incubated aerobically at 22° to 25°C for three hours and then stored at 4°C. Samples of the stock suspension were removed and diluted as required for testing. The stock suspension was renewed weekly from freshly thawed pellets.

The ascorbic acid used in these tests was USP grade, produced by Merck & Co. All other chemicals were reagent grade, except as noted.

All solutions and suspensions were prepared from distilled water, which was sometimes purified by passage through a Barnstead* demineralizer cartridge. Solutions of sodium ascorbate were sometimes depleted of their heavy metal contents by treatment with the sodium form of Duolite C-3** or Dowex 50*** cation-exchange resins.

B. METHODS

1. Standard Assay of Ascorbate Toxicity

The stock cell suspension was diluted in water to a level of 10×10^{4} viable cells per milliliter. One-tenth milliliter of this suspension was added to 9.9 milliliters of a sodium ascorbate solution, yielding a test suspension containing 10×10^{2} cells per milliliter in one per cent sodium ascorbate at pH 7.0. As rapidly as possible (within 35 to 45 seconds) the test suspension was assayed for its viable cell population by spreading 0.1-ml samples on the surface of nutrient agar plates. The test suspensions were held at 22° to 24°C (room temperature) for 15 minutes and then re-assayed for population levels by the same surface plating technique. The viable cell population of the assayed samples was assumed to equal the numbers of colonies developed upon these plates after 16 to 20 hours' incubation at 30°C. The dilution and plating protocol was so arranged that 100 colonies per plate was the anticipated level of count equivalent to 100 per cent recovery or undiminished survival. In the tabulated data, this recovery level is indicated by "/"; colony counts of five or less (usually zero) are represented by "-" signs.

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* Barnstead #0802 standard ion-exchange resin cartridge, manufactured by Barnstead Still and Sterilizer Co., Boston, Massachusetts.
** Manufactured by the Chemical Process Co., Redwood City, California.
*** Manufactured by the Dow Chemical Co., Midland, Michigan.

The standard operating procedure was varied in the following ways:

(a) Sodium ascorbate solutions were prepared in the presence of additional solutes.

(b) Traces of contaminating heavy metal ions were removed from sodium ascorbate solutions by batch-type treatment with Dowex 50 or Duolite C-3 cation exchange resins in the sodium form, as follows: Five grams of resin were added to 100 milliliters of one per cent sodium ascorbate in a 250-ml beaker and the mixture was held at 22° to 24°C for not less than 15 minutes. The ascorbate was then separated from the resin by centrifugation or decantation.

(c) Sodium ascorbate solutions were freed of traces of contaminating heavy metal ions by extraction with diphenylthiocarbazone (dithizone) in CCl_4 solution. The ascorbate and dithizone solutions were shaken together in a separatory funnel for at least 15 minutes, then the CCl_4 layer was discarded. Consecutive extractions of the ascorbate with CCl_4 were performed, until a colorless CCl_4 phase was obtained. The ascorbate solution was then drawn off and held under negative pressure in a vacuum flask until all residual CCl_4 was volatilized.

(d) In some experiments, cells were exposed to solutions of one per cent sodium ascorbate both in the presence and in the absence of air. The anaerobic exposure of the cells was executed in units such as that shown in Figure 1. Paired units of identical solute content were prepared for each experimental treatment, the viable cell population of one unit being determined at "zero time" and the other after 15 minutes' exposure. The units were prepared for use as follows:

(1) Either 9.4 or 9.8 milliliters of solution, containing 100 milligrams of ascorbic acid neutralized to pH 7 with NaOH, was placed in Compartment A (Figure 1).

(2) Either 0.1 or 0.5 milliliter of water or test solution was placed in Compartment B. The combined volume of the contents of Compartments A and B was always equal to 9.9 milliliters.

(3) One-tenth milliliter of cell suspension containing 10×10^3 cells in distilled water was placed in Compartment C.

(4) The ground-glass joints of the sections of each unit were lubricated with high-vacuum grease. Each of the units was assembled, evacuated to less than five microns pressure with a Cenco-Megavac pump protected with a cold (-78°C) trap, and then sealed. During this degassing process, the flasks became quite cold. Each unit was allowed at least 15 minutes to permit it to warm to room temperature after its evacuation was completed.

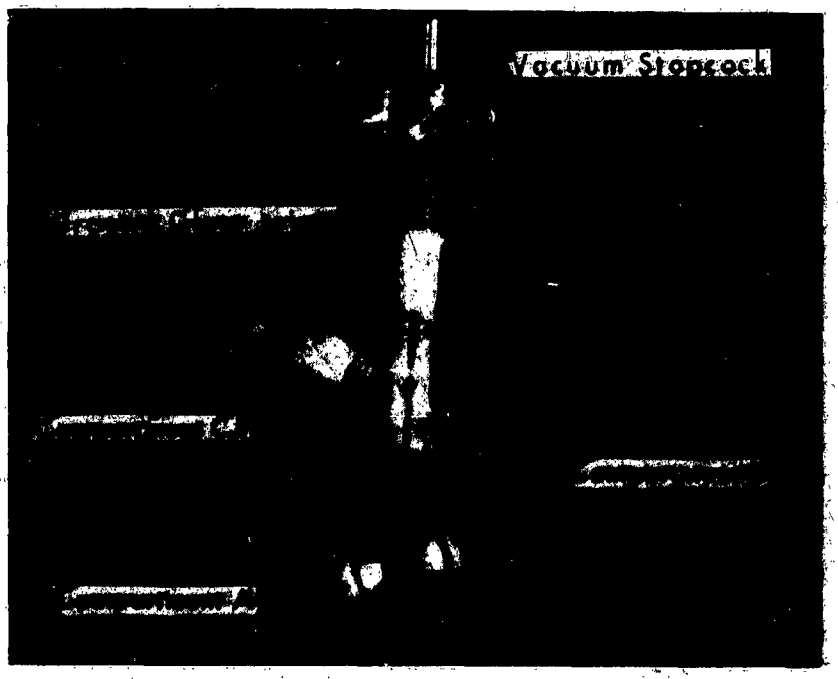

Figure 1. Apparatus for Exposing S. marcescens to Solutions of Ascorbates in vacuo. (FD Neg C-6473)

(5) The contents of Compartment B were tipped into Compartment A.

Tests were performed with the evacuated units by the following procedure:

(a) Using Unit 1 of a pair, the cell suspension in Compartment C was tipped into the mixed solution in Compartment A.

(b) As rapidly as possible (within 20 seconds) the vacuum in the system was broken and 0.1-ml samples of the cell suspension removed and spread on agar plates. These were considered as "zero time of exposure" samples.

(c) The 15-minute samples were obtained by using Unit 2, which was treated in the same way as Unit 1, except that the interval between mixing the solutions and breaking the vacuum was extended to 15 minutes.

2. Quality of the Data

Solutes were tested in groups to determine their toxicity toward S. marcescens at low population densities. Six to twelve solutes were assayed per group in aerobic tests, and three to five solutes per group in anaerobic tests. Each test group always included reference solutions that verified one or more of the following variables: (a) the population level of the cell suspension, (b) the nontoxicity of purified sodium ascorbate solutions, and (c) the toxicity of purified sodium ascorbate solutions to which copper sulfate was added.

Experimental observations were considered to be valid data only when every solute, in a group under test, yielded the same experimental outcome in three consecutive replicate trials.

3. Oxygen Uptake Studies

The oxygen uptake rates of suspensions of S. marcescens and solutions of ascorbic acid were determined in the Warburg apparatus by standard techniques[9] except that no phosphate or other buffers were added to the cell suspensions.

III. RESULTS

As shown in Table I, one per cent sodium ascorbate apparently killed S. marcescens within 15 minutes in suspensions containing 10×10^2 cells per milliliter, whereas ascorbate was nontoxic after 24 hours to suspensions containing 20×10^9 cells per milliliter. Sodium ascorbate showed a rapid oxygen uptake rate only in the suspensions where it was toxic. The toxicity of ascorbate and its rate of auto-oxidation thus appeared to be correlated. This possible interaction, therefore, was investigated further.

TABLE I. CORRELATION BETWEEN OXYGEN UPTAKE RATE OF SODIUM ASCORBATE AND ITS TOXICITY TO S. MARCESCENS

ADDED SOLUTES IN CELL SUSPENSION[a]	10×10^2 CELLS PER ML		20×10^9 CELLS PER ML	
	Oxygen Uptake Rate, µl/min	Viable Cell[b] Survival After 15 Min and After 24 Hr, per cent	Oxygen Uptake Rate, µl/min	Viable Cell[b] Survival After 15 Min and After 24 Hr, per cent
None (water)	0	100	0.6	100
1% Sodium Ascorbate	4.3	0	1.3	100

a. All cell suspensions prepared with ordinary distilled water.
b. All suspensions yielded 100 per cent survival when plated at "zero time." Survival levels after 15 minutes and 24 hours were identical.

The auto-oxidation of ascorbic acid solutions was reported by the Merck Index[10]* to be catalyzed by traces of iron (Fe) and copper (Cu). This effect was verified experimentally, as indicated by the data in Table II. The application of metal-sequestering treatments to ascorbate solutions suppressed their auto-oxidation and also eliminated their toxicity in suspensions containing 1000 cells per milliliter, as shown in Table III. (All suspensions mentioned hereafter contained 1000 cells per milliliter.) The detoxification of ascorbate solutions by dithizone extraction or treatment with ion-exchange resins apparently indicated that oxidation of ascorbate was responsible for its toxicity; removal of the metals catalyzing such oxidation also eliminated toxicity.

* Page 106.

TABLE II. CATALYSIS OF AUTO-OXIDATION OF SODIUM ASCORBATE SOLUTIONS[a] BY SALTS OF COPPER AND IRON

PER CENT OF SALT ADDED	OXYGEN UPTAKE RATES, μl O_2/min		
	None	$FeSO_4 \cdot 7H_2O$	$CuSO_4 \cdot 5H_2O$
0	0.6-1.2		
0.00001		0.4-0.7	3.3-4.1
0.001		2.8-2.9	15.4-20.3

a. One per cent solutions prepared with distilled water redistilled from an all-glass still.

TABLE III. SUPPRESSION OF THE AUTO-OXIDATION AND THE TOXICITY OF SODIUM ASCORBATE[a] BY METAL-SEQUESTERING TREATMENTS

METAL-SEQUESTERING PROCESS	SURVIVAL[b] OF CELLS AFTER 15 MINUTES	OXYGEN UPTAKE RATE OF TREATED ASCORBATE IN ABSENCE OF ADDED CELLS, μl/min
By Addition of Solutes[c]		
None	-	4.3
0.5% Na versenate (pH to 7.0 w/NaOH)	∮	0.4
0.4% Na arsenite (pH to 7.0 w/H_2SO_4)	∮	0.5
0.1% Na diethyldithiocarbamate	∮	0.2
0.001% NaCN	∮	0.6
<0.1% (Sat) 8-hydroxyquinoline	∮	0.5
<0.1% (Sat) dithizone	∮	0.2
0.1% thiosemicarbazide	∮	0.6
0.1% thiourea	∮	0.3
By Extraction[d]		
With dithizone - CCl_4	∮	0.5
With Dowex 50 resin, Na form	∮	2.1
With Duolite C-3 resin, Na form	∮	3.6

a. In suspensions containing 1000 S. marcescens cells per ml and 1% ascorbic acid brought to pH 7 with NaOH; these were prepared with ordinary distilled water.
b. ∮ = 100% survival.
- = 0 (<5%) survival.
Viable cell survival in all suspensions was "∮" after "zero time."
c. Added to ascorbate during preparation of cell suspensions.
d. Extraction performed upon sodium ascorbate solutions before they were used to prepare suspensions.

Suspensions containing ascorbate could be prepared, however, that auto-oxidized rapidly but were also nontoxic. As shown in Table IV, these suspensions contained (a) the NH_4 salt of ascorbic acid, or (b) sodium ascorbate plus dipyridyl or aminoguanidine. Rapidly oxidizing ascorbate solutions were thus not invariably toxic, and so the auto-oxidation of ascorbate was apparently not responsible for its toxicity. On this basis, study of the oxygen uptake rates of ascorbate solutions was not continued.

TABLE IV. DISSOCIATION OF THE AUTO-OXIDATIVE AND THE TOXIC PROPERTIES OF SODIUM ASCORBATE[a/]

SOLUTES IN CELL SUSPENSION	VIABLE CELL SURVIVAL[b/] AFTER 15 MIN	OXYGEN UPTAKE RATE OF SOLUTION, µl/min
None	/	0
1% Ascorbic acid, Na salt	-	4.3
1% Ascorbic acid, Na salt plus 0.01% dipyridyl	/	11.6
1% Ascorbic acid, Na salt plus 0.5% aminoguanidine H_2CO_3	/	4.1
1% Ascorbic acid, NH_4 salt	/	15.0
0.5% Ascorbic acid, Na salt plus 0.5% ascorbic acid, NH_4 salt	/	12.0

a. Toxicity determined in suspensions containing 1000 S. marcescens cells per ml and 1% ascorbic acid brought to pH 7 with NaOH or NH_4OH. Measurements of auto-oxidation rates made on similar solutions not containing cells. All suspensions and solutions prepared with ordinary distilled water.

b. / = 100% survival.
- = 0 (<5%) survival.
Viable cell survival in all suspensions at "zero time" was "/."

Since metal-sequestering treatments eliminated the toxicity of ascorbate solutions, one would predict that when the active metal ions were added back to the detoxified solution the toxicity of ascorbate would be restored. Accordingly, the toxicity of a number of heavy-metal ions to S. marcescens was determined in the presence and absence of ascorbate. The results of these tests are shown in Table V. Most of the tested salts were nontoxic in the presence or absence of ascorbate. At 0.001 per cent concentration, only $AgNO_3$ and $CuSO_4 \cdot 5H_2O$ were lethal to cells suspended in water. The toxicity of Ag was abolished in the presence of ascorbate, presumably because of the complete conversion of ionized to metallic Ag.

Concentrations of 0.001 per cent $CuSO_4 \cdot 5H_2O$ remained bactericidal in the presence of one per cent sodium ascorbate. Lesser concentrations of $CuSO_4 \cdot 5H_2O$ proved to be toxic only in the presence of sodium ascorbate. Solutions containing 0.00001 per cent $CuSO_4 \cdot 5H_2O$ (equal to 0.025 ppm of Cu) plus one per cent sodium ascorbate were found to be bactericidal, although each of the solutes when tested individually was nontoxic. This toxic mixture will be termed hereafter Cu-ascorbate.

TABLE V. EFFECTS OF METAL SALTS UPON THE VIABILITY OF S. MARCESCENS IN SUSPENSIONS CONTAINING 1000 CELLS PER ML

ADDED METAL SALT	VIABLE CELL SURVIVAL[a] AFTER 15 MINUTES' EXPOSURE		
	In Water	In 1% Na Ascorbate[b] Solution	
		Without Added Copper	Plus 0.00001% $CuSO_4 \cdot 5H_2O$
None	/	/	-
0.001% $AgNO_3$	-	/	-
0.001% $NiSO_4 \cdot 7H_2O$	/	/	-
0.001% $CoCl_2 \cdot 6H_2O$	/	/	-
0.001% $MnSO_4 \cdot H_2O$	/	/	-
0.001% $Cr(NO_3)_3 \cdot 9H_2O$	/	/	-
0.001% $ZnSO_4 \cdot 7H_2O$	/	/	-
0.001% $Ba(C_2H_3O_2)_2 \cdot H_2O$	/	/	-
0.001% $Ca(NO_3)_2 \cdot 4H_2O$	/	/	-
0.001% $MgSO_4 \cdot 7H_2O$	/	/	-
0.001% $3CdSO_4 \cdot 8H_2O$	/	/	-
0.001% $Pb(C_2H_3O_2)_2 \cdot 3H_2O$	/	/	-
0.001% $SnCl_2 \cdot 6H_2O$	/	/	-
0.001% $LiSO_4 \cdot H_2O$	/	/	-
0.001% $Al_2Cl_6 \cdot 12H_2O$	/	/	-
0.001% $CuSO_4 \cdot 5H_2O$	-	-	
0.0001% $CuSO_4 \cdot 5H_2O$	Variable[c]	-	
0.00001% $CuSO_4 \cdot 5H_2O$	/	-	
0.000001% $CuSO_4 \cdot 5H_2O$	/	/	
0.001% $FeSO_4 \cdot 7H_2O$	/	/	/

a. / = 100% survival.
- = 0 (≤5%) survival.
Viable cell survival in all suspensions was "/" after "zero time."

b. Ascorbic acid solutions were all adjusted to pH 7.0 with NaOH. Some of these solutions were prepared with ordinary distilled water and extracted with dithizone - CCl_4; others were prepared with water passed through a Barnstead demineralizer cartridge. Both types of ascorbate solutions were equally nontoxic.

c. Eight to 30 per cent survival in replicate trials.

The toxicity of Cu-ascorbate was abolished by Fe salts at concentrations down to 0.001 per cent. This effect was not obtained in the presence of cations other than Fe, as shown in Table V. A number of nitrogenous compounds possessing ionized amino or ammonium groups also rendered Cu-ascorbate nontoxic. These compounds included all of the alpha-amino acids, guanidine, hydrazine, β-alanine, and $(NH_4)_2SO_4$. Nitrogenous compounds possessing unionized amino groups (urea, acetamide) or multi-substituted NH_4 groupings [$(CH_3)_4NOH$, $(C_2H_5)_4NOH$, and $(CH_3)_2NH$] were unable to detoxify Cu-ascorbate. These effects are summarized in Table VI.

As shown in Table VII, the sodium salts of anions such as citrate, picolinate, oxalate, phosphate, and halogen acids also interfered with the toxicity of Cu-ascorbate. These anions were all reported to form complexes with Fe ions.[11-13*] The sodium salts of other acids not possessing this property, such as nitrate, glucuronate, and sulfate, did not modify the toxicity of Cu-ascorbate; neither did a plasmolytic (ten per cent) or non-plasmolytic (one per cent) concentration of sucrose.

Finally, experiments were performed to define the relationship between the presence of oxygen and the toxicity of Cu-ascorbate. The results of this work are shown in Table VIII. Whether or not oxygen was present, (a) Cu-ascorbate was toxic but ascorbate alone was not, and (b) the bactericidal effects of Cu-ascorbate were suppressed in the presence of 0.5 per cent NaCl or 0.5 per cent $(NH_4)_2SO_4$. The ability of 0.001 per cent Fe SO_4 to protect S. marcescens from the toxic effects of Cu-ascorbate, however, disappeared when oxygen was removed from the reaction system.

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* Pages 9, 38-39, and 588.

TABLE VI. EFFECTS OF ADDED NITROGENOUS COMPOUNDS UPON THE TOXICITY[a] OF ASCORBIC ACID PLUS COPPER[b]

SOLUTES NOT SUPPRESSING TOXICITY AT 0.5% CONCENTRATION	SOLUTES SUPPRESSING TOXICITY AT 0.5% CONCENTRATION
Urea	Glycine
Acetamide	Alanine
$(CH_3)_4$•NOH sulfate	Leucine
Trimethylamine oxide	Norleucine
Dimethylamine sulfate	Isoleucine
$(C_2H_5)_4$•NOH sulfate	Valine
	Cysteine HCl
	Methionine
	Serine
	Threonine
	Phenylalanine
	Tyrosine[c]
	Tryptophane
	Proline
	Hydroxyproline
	Histidine HCl
	Lysine HCl
	Arginine HCl
	Aspartic acid, Na salt
	Glutamic acid, Na salt
	Creatine hydrate
	Glutathione
	$(NH_4)_2SO_4$
	Guanidine CO_3
	Guanidine HCl
	Glycocyamine
	Hydrazine sulfate
	β-alanine

a. Toxicity defined as the killing, within 15 minutes, of more than 95% of the cells in suspensions containing 1000 S. marcescens cells per ml.

b. One per cent ascorbic acid, pH adjusted to 7.0 with NaOH and extracted with Duolite C-3 resin, Na form. $CuSO_4 \cdot 5H_2O$ added sufficient to yield 0.00001% in final reaction mixture.

c. Saturated solution used instead of 0.5% concentration.

TABLE VII. EFFECTS OF MISCELLANEOUS SOLUTES IN SUPPRESSING THE TOXICITY[a] OF COPPER-ASCORBATE[b]

ADDED COMPOUNDS NOT SUPPRESSING TOXICITY	ADDED COMPOUNDS SUPPRESSING TOXICITY
1.2% Na_2SO_4	0.3% NaCl
1% Sucrose	0.5% NaBr
10% Sucrose	0.5% NaI
1% Glucuronic acid, Na salt	0.5% Na citrate
0.5% $NaNO_3$	0.5% Na oxalate
0.5% NaF	0.5% Na picolinate
0.5% Na thiocyanate	0.5% Na H_2PO_4/Na_2HPO_4
	0.5% Sodium thioglycollate
	0.5% Sodium sulfite
	0.5% Sodium thiosulfate
	0.5% Sodium hydrosulfide
	0.5% Sodium hydrosulfite

a. Toxicity defined as the killing, within 15 minutes, of more than 95% of the population in suspensions containing 1000 S. marcescens cells per ml.
b. One per cent ascorbic acid adjusted to pH 7.0 with NaOH, prepared with water passed through a Barnstead demineralizer cartridge. $CuSO_4 \cdot 5H_2O$ added sufficient to make a 0.00001% final concentration.

TABLE VIII. SUPPRESSION OF THE TOXICITY[a] OF COPPER-ASCORBATE BY INDICATED SOLUTES IN THE PRESENCE AND ABSENCE OF AIR

SOLUTE CONTENT OF CELL SUSPENSION, 1% Ascorbic Acid, Na Salt,[c] Plus	VIABLE CELL SURVIVAL[b] AFTER 15 MINUTES' EXPOSURE	
	Aerobical	Anaerobical
No Added Solutes	+	+
0.00001% $CuSO_4 \cdot 5H_2O$	-	-
0.00001% $CuSO_4 \cdot 5H_2O$ + 0.001% $FeSO_4 \cdot 7H_2O$	+	-
0.00001% $CuSO_4 \cdot 5H_2O$ + 0.5% NaCl	+	+
0.00001% $CuSO_4 \cdot 5H_2O$ + 0.5% $(NH_4)_2SO_4$	+	+

a. Toxicity defined as the killing, within 15 minutes, of more than 95% of the population of suspensions containing 1000 S. marcescens cells per ml.
b. + = 100% survival; - = no (<5%) survival.
Viable cell survival in all suspensions was "+" at "zero time."
c. Prepared with distilled water passed through a Barnstead demineralizer cartridge.

IV. DISCUSSION

A. TOXICITY OF ASCORBATE AND ITS REVERSAL

1. Metal-Sequestering Treatments

This investigation was begun in an attempt to explain the observation that neutral solutions of ascorbate were toxic to sparsely but not to densely populated suspensions of S. marcescens cells. The data clearly showed that neutral solutions of ascorbate were nontoxic, but they potentiated the antibacterial effects of copper salts; Cu-ascorbate, rather than ascorbate, was the truly toxic material. Cu-ascorbate is an active reducing agent, as shown by its rapid auto-oxidation rate, and so it might have killed S. marcescens by reducing some indispensable cellular component into a nonfunctional state. Ascorbate solutions containing only trace amounts of Cu may have been actively antibacterial because Cu^{+}, the postulated active toxicant, was regenerated by ascorbate as fast as it was oxidized, by reaction with the cells, to form Cu^{++}. The fact that Cu-ascorbate killed cells equally well in the presence or absence of oxygen indicated that its toxicity could not be due to compounds (such as H_2O_2) formed as a result of the aerobic oxidation of ascorbate. Metal-sequestering solutes and treatments undoubtedly suppressed the auto-oxidation and the toxicity of sodium ascorbate solutions by removing the copper ions necessary to catalyze both these reactions.

2. Iron Salts

Of all the metal salts tested, only iron salts protected cells against the toxicity of Cu-ascorbate, and then only in the presence of air. Fe and Cu ions both catalyze the auto-oxidation of ascorbate, but oxidized Fe forms a relatively stable complex with ascorbate. The stability of this complex was responsible for the relatively inefficient catalysis of ascorbate auto-oxidation by Fe. The Fe^{+++}-ascorbate complex forms a violet solution similar to that formed by ferric salts with cysteine[14]/* or glutathione[14]/** or sulfosalicylic acid;[10]/*** the Fe^{++}-ascorbate complex, formed anaerobically, is colorless.

With these observations in mind, it is postulated that the reactive Cu^{+} ions may irreversibly reduce an iron atom in an indispensable cellular component from Fe^{+++} to Fe^{++}, thereby inactivating the cell component and so causing death. The Fe^{+++}-ascorbate molecules may provide an equally reactive substrate for the Cu^{+} ions to reduce, thus exerting a sparing effect on the sensitive cell components. When S. marcescens was exposed to Cu-ascorbate in the presence of iron salts in vacuo, no Fe^{+++}-ascorbate was produced, no alternative substrate was provided to divert the reducing activity of Cu^{+}, and the toxic effects of Cu-ascorbate were unmodified.

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* Pages 34-35.
** Pages 42-43.
*** Page 1002.

3. Metal-Complexing Solutes

The remaining solutes that eliminated the toxicity of Cu-ascorbate for S. marcescens are listed in Tables IV, VI, VII. These included amino acids, hydrazine and guanidine derivatives, ammonium compounds, and the sodium salts of miscellaneous acids. All of these compounds were reported to form complexes of limited stability with ions of Fe, Ni, Co, and Cu, the metals of the first transition series. Complexing agents such as dipyridyl or ammonium ions did not disrupt the Cu-ascorbate complex by sequestering Cu, as shown by their inability to suppress the auto-oxidation of ascorbate solutions. Although such solutes did not interfere with the reaction between Cu-ascorbate and aerobic oxygen, they must have interfered somehow with the reaction between Cu-ascorbate and the cells.

It was postulated previously that Fe^{+++}-ascorbate maintained cell viability by acting as an alternate substrate for the reducing action of Cu-ascorbate, a sparing action thereby being exerted upon hypothetical vital cellular Fe^{+++} atoms. With this thought in mind, it is suggested that the solutes presently under discussion formed complexes with the cellular Fe^{+++} atoms, thereby preventing Cu-ascorbate from reacting with them. The presence or absence of oxygen had no effect on the protective activity of solutes such as $(NH_4)_2SO_4$ or NaCl, an observation consistent with this hypothesis.

B. NONTOXICITY OF ASCORBATE TO DENSELY POPULATED CELL SUSPENSIONS

The auto-oxidation of ascorbate was suppressed in the presence of 20×10^9 cells per milliliter. Therefore, it appears that the cells, and/or the solutes in their suspending fluid, sequestered the traces of Cu present in the ascorbate solutions, thereby disrupting the Cu-ascorbate complex and abolishing its toxicity.

C. RESULTS OF EARLIER WORKERS

Previous investigators had attributed the toxic effects of ascorbic acid either to its auto-oxidation or to a compound generated during this process. The data presented here have indicated that the apparent toxicity of ascorbate is created by the presence of copper, not oxygen. Ericsson and Lundbeck[6,7] were the only previous workers to report the antibacterial interactions of copper and ascorbate. They exposed bacteria to solutions of both sodium ascorbate and copper sulfate plus H_2O_2, in order "to determine the effects of the oxidation of ascorbic acid upon viability of the organisms." These solutes, at concentrations that were individually nontoxic, proved bactericidal in combination to the following organisms: "Pneumococcus Type I; Streptococcus viridans, hemolyticus, and fecalis;

Staphylococcus albus and aureus; E. coli; B. proteus; Ps. aeruginosa; C. diphtheriae; S. typhimurium, and H. pertussis." The only species resistant to this treatment were M. tuberculosis and the spore-forming B. subtilis. Cu-ascorbate thus appeared to have generalized antibacterial effects. Ericsson and Lundbeck, however, were convinced that the toxicity of ascorbate was due to its aerobic oxidation. They did not attempt to verify this postulate by performing anaerobic toxicity tests, and so they obtained no insight into the mechanism whereby ascorbate killed bacteria. They stated only that no stable oxidation product of ascorbate was responsible for its toxicity, a conclusion in agreement with ours.

A system of copper toxicity very similar to the one reported here was recently described for E. coli and A. aerogenes by Gorini.[15] The growth of mutant strains of these organisms anaerobically (but not aerobically) was greatly or indefinitely delayed by the presence of traces of copper. The copper was presumably introduced in the distilled water; its effects were eliminated in the presence of air or SH-containing compounds such as cystine and cysteine. These data were entirely consistent with our observation that the toxicity of copper may be greatly enhanced under reducing conditions.

D. SOURCE OF COPPER CONTAMINATION

Solutions of sodium ascorbate were rendered nontoxic to S. marcescens by extraction with cation exchange resins or with dithizone, presumably because these materials removed traces of copper from the solutions. Laboratory techniques were modified as follows in efforts to discover and eliminate the source of copper contamination: (a) dilution and plating procedures were usually performed with unplugged, unwrapped pipettes sterilized by dry heat in copper cans; enameled dressing trays were substituted for the copper cans; (b) solutions were prepared from the ordinary single-distilled water, purified by passage through a Barnstead demineralizer cartridge. Together, but not separately, these modifications permitted the direct preparation of ascorbate solutions that were nontoxic under the standard conditions of test. It was, therefore, concluded that the previous copper contamination came both from the distilled water and from the copper pipette cans; copper was not present in toxic concentration on the cells or in the reagent chemicals.

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